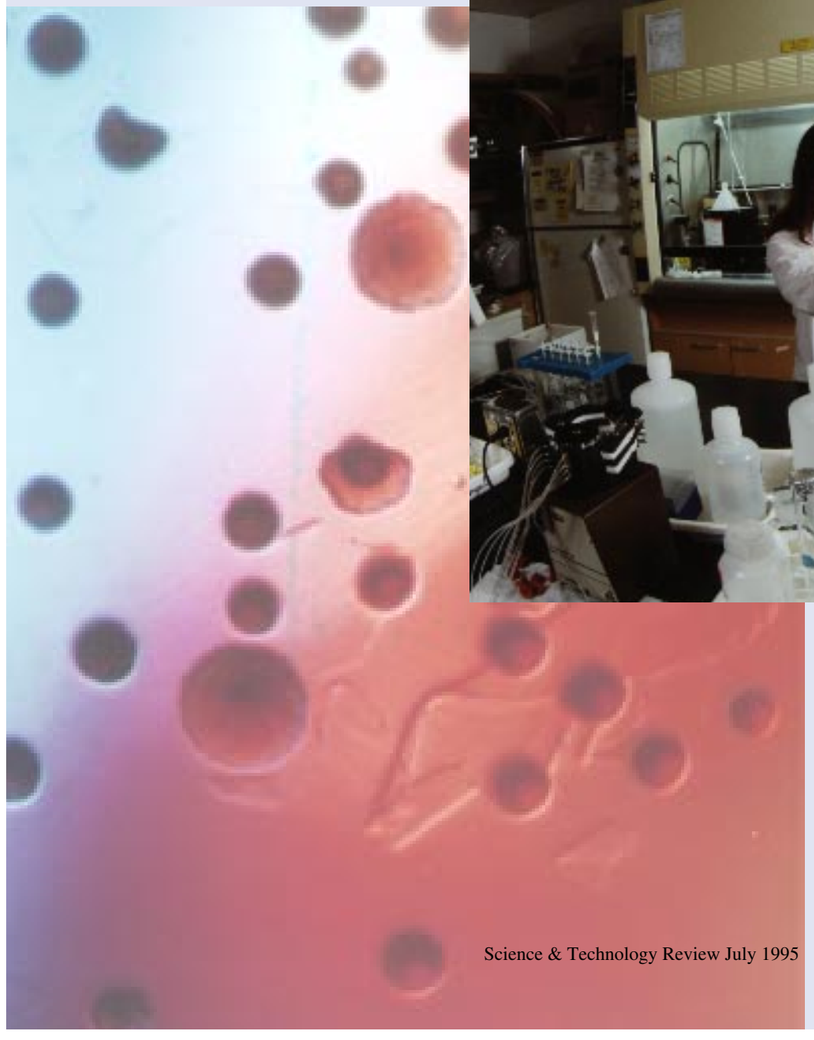
Food Mutagens:
The Role of Cooked Food in Genetic Changes



When food derived from muscle is heated, potent mutagens are produced. For nearly two decades, LLNL researchers have studied the formation of toxic mutagenic compounds in red meats and other foods containing protein. This report, the first of two installments, focuses on the identification of food mutagens and measurement of their abundance in cooked foods as a function of cooking temperature and time.

ACCORDING to a common, rather simplistic notion, we are what we eat. On a far more empirical level, epidemiological studies reveal a connection between diet and adverse health consequences. Many observed differences in cancer rates worldwide, including incidences of colon and breast cancer, are linked to variations in human diets.

Strong evidence suggests that mutations are the initiating events in the cancer process. In other words, the complex sequence of cellular changes ultimately leading to malignant tumors is thought to begin with structural changes—mutations—within the molecular units that make up the genes.

For 17 years, LLNL researchers have been investigating certain biologically active compounds in foods that can trigger tumors in animals, at least after exposure to high concentrations, by producing cellular mutations.

At first glance, identifying the mutagens that might put us at risk and understanding how they affect the body appear to be simple matters. In fact, the opposite is true. Consider just a few of the questions that must be addressed to understand the entire picture of diet-induced mutations and possible links to cancer. Exactly what compounds in foods are dangerous, how are the compounds formed during cooking, in what amounts are they present after cooking, and how toxic or cancer-causing are they? What chemical changes take place metabolically at the molecular level after the mutagenic substances are consumed? For example, what role do metabolic enzymes play, how is DNA affected, and how might tumors be triggered in the body's somatic cells? What chemical, tissue, animal, and human models might be useful to estimate risk to the human population? Are all people affected similarly, or are some resistant to cancer-causing effects? If people vary in cancer incidence, what accounts for the differences in susceptibility?

Table 1. Some of the required interdisciplinary research, analytic methods, and tools needed to understand the possible connection of mutagens in cooked food to cancer.

Issues	Research required	Analytic methods and tools
• What cooked foods contain mutagens? • What are the mutagenic compounds? • What amounts are produced?	• Chemical extraction and purification • Identification and quantification • Proof of structure • Synthesis of isomers	• Gas chromatography (GC) • Liquid chromatography (LC) • Mass spectrometry (MS) • High-resolution mass spectrometry (HRMS) • Nuclear magnetic resonance (NMR) spectrometry • Ames/*Salmonella* test • Monoclonal antibodies
• By what mechanism are mutagens formed during cooking?	• Study precursors and reaction conditions in chemical models • Aqueous vs dry heating • Vary cooking temperature	• Modeling mutagens from – creatine – creatinine – amino acids – sugars • Heavy isotope incorporation
• How potent (mutagenic) are the compounds?	• Mutagenicity research (e.g., use chemical to induce mutations, and count frequency of mutant cells or chromosomal changes)	• High-performance liquid chromatography (HPLC) • Ames/*Salmonella* test • Animal mutation studies – Mice – Chinese hamster ovary (CHO) cell cultures
• How are mutagens activated metabolically?	• Study chemical intermediates (bioactivation pathways) • Modulate metabolism in cell models • Radioactive labeling	• Cell models – Mammalian cell systems – Bacterial cell cultures • Enzyme inhibitors
• How is DNA affected?	• DNA damage and repair • DNA binding analysis • Data adduct analysis	• Computational chemistry analysis • ^{32}P-postlabeling of DNA adducts • Accelerator mass spectrometry (AMS) • Models – Whole animals (*in vivo*) – Animal cells in culture (*in vitro*) – Bacterial assays
• How are tumors induced?	• Carcinogenicity research (e.g., assess tumor induction in various tissues in laboratory animals)	• Animal models – Monkeys – Rats – Mice
• What are the health risks from exposure? • What people are affected? • Who is most at risk?	• Dose-response assessment in humans • Adduct formation as an indicator of exposure • Risk assessment • Extrapolation from animal studies	• ^{32}P-postlabeling of DNA adducts • Accelerator mass spectrometry • Epidemiology

Clearly then, isolating, identifying, and assessing the biological activity of mutagenic compounds in food is a difficult problem requiring extensive effort. Table 1 is an overview of some of the research issues addressed and analytic methods used in this field of investigation. This series of articles focuses on the first five questions under "Issues" listed in Table 1. A second installment in *Science and Technology Review* will address the remaining issues.

A simple analogy can help put a key feature of our work into perspective. The compounds we have been investigating for nearly two decades—the aromatic heterocyclic amines—are present in cooked foods at very low levels, in the range of about 0.1 to 50 parts per billion. Isolating material at the part-per-billion level is equivalent to pouring a jigger of Scotch into the hold of a full supertanker and then trying to retrieve it again. Although the compounds we study are present in very small amounts, they are also the most mutagenic compounds ever found, and they produce tumors in mice, rats, and monkeys. Such knowledge, combined with the fact that these compounds are present in many foods characteristic of the Western diet and that certain diets are known to influence tumors at several body sites, gives our research an extra sense of urgency.

LLNL's Approach

The single aspect that best characterizes our research on food mutagens and carcinogens—and sets our work apart from almost all other efforts around the world—is its multidisciplinary nature. Biomedical scientists at LLNL routinely collaborate with investigators working in analytical chemistry, synthetic chemistry, quantum chemistry, physics, the environmental sciences, and forensics (Figure 1). Our research requires tools such as accelerator mass spectrometry and nuclear magnetic resonance spectrometry, to name a few. The Laboratory is one of the few places that brings together the broad expertise and state-of-the-art analytic tools required to fully understand each important aspect of the problem of mutagens and carcinogens in the human diet. The way we became involved in this field of research has much to do with our role as a national laboratory with interdisciplinary research programs.

Mutagens are the damaging agents that can structurally change the molecular units that make up the genes (that is, the genetic material, DNA) or the relation of one chromosome to another. For many years, LLNL investigators have been studying some of the ways that x rays, ultraviolet light, and some chemicals in the environment can act as mutagens. Carcinogens are agents that incite the development of a cancerous tumor or other malignancy. Some 80 to 90% of mutagenic substances are also carcinogenic. More than 50 years ago, scientists painted the skin of mice with extracts from heated animal muscle and found that the extracts were carcinogenic, but the research went no further.

By the early 1970s, Bruce Ames at the University of California, Berkeley, had developed a biological test to measure the mutagenic potency (mutagenicity) of substances.[1*] In the late 1970s, T. Sugimura, who directed research at the National Cancer Center in Tokyo, applied the Ames method and published the fact that smoke condensate from cooking and the charred surface of broiled fish and beef were mutagenic.[2] One year later, Barry Commoner, working at Washington University, St. Louis, used the Ames method to show that cooking temperature and time affect the formation of mutagens in food.[3]

The news that cooking amino acids (the building blocks of proteins) and muscle-containing foods could be dangerous triggered considerable scientific interest around the world. In 1978, biomedical researchers at LLNL were working on the problem of mutagenic chemicals produced by oil shale retorting and coal gasification. Because of our combined expertise in chemical analysis (including different types of chromatography and spectrometry), biological analysis (including the Ames mutation assay), and our emerging program in genetics and toxicology, we received a multiyear contract from the National Institute of Environmental Health Science (NIEHS) to take a detailed look into the problem of food mutagens. As it turns out, what happens when oil shale and coal are heated is not so different from some of

Figure 1. Cyndy Salmon, one of the researchers in the LLNL food mutagen research group, pours a cooked food sample into an extraction tube to prepare it for subsequent analysis.

*All references are at the conclusion of the third part of this installment on p. 25.

the chemical processes that occur when a hamburger is cooked.

Our work on food mutagens also parallels our interest in the mechanisms by which pesticides and many other toxic chemicals can elicit adverse biological responses. For example, benzo[a]pyrene is a widely studied pollutant found in combustion products, and it has been isolated from burned fat and cigarette smoke. However, this compound becomes carcinogenic only after it interacts with DNA following oxidation by metabolic enzymes. The production of such enzymes and their roles in changing the chemical reactivity of compounds are part of the body's normal biological response to certain foreign substances. We are learning that similar "metabolic activation" takes place before food mutagens become harmful.

Today, our research is funded primarily by the National Cancer Institute, with additional support from the Laboratory Directed Research and Development program and other sources. There are approximately 50 other prominent research teams worldwide studying the heterocyclic amines. However, except for one other program in Japan, ours is the only team that brings a truly multidisciplinary approach to the problem of understanding mutagens and carcinogens associated with cooked food and their consequences at the cellular, genetic, and molecular levels.

A Problem of Strategy

Strictly speaking, it is inaccurate to say that cooked foods contain mutagens. More precisely, certain cooked foods contain premutagenic substances (promutagens) that are metabolized by enzymes naturally present in body tissues, leading to the formation of one or more reactive mutagenic substances. Conventionally, however, "promutagen" and "mutagen" are used synonomously, and we have followed that practice here unless the point being made about the research demands a precise distinction.

At the outset of our research, we were faced with problems of strategy. Studying substances that are present at very low concentrations imposes many research constraints. If we focused on only a few foods, as seemed wise, then our results and their implications for public health might be misinterpreted. Instead, we decided to examine the foods that are the principal sources of cooked protein: meats (any muscle-containing food, including fish), eggs, beans, cheese, and tofu. Whereas we initially focused on meats, especially fried beef, we have now expanded the range of foods to include cooked breads and grain products, heated flour from many different plant sources, and meat substitutes.

Over the years, our research has also evolved from relatively simple concepts and approaches to more sophisticated ones. Initially, we had to identify the mutagenic compounds in heated foods because many were not known (that is, neither synthesized nor analyzed). Thus, we focused our efforts on identifying the chemical composition and structure of mutagens, assessing how different cooking procedures affect the formation of mutagens, and determining the amount (abundance) of the mutagenic products. Even though chemical identification and quantification are still important activities, our work has expanded to include many other aspects of the problem.

For example, we developed techniques to help us learn how mutagens are metabolized in the body. We use animals as models to understand complex metabolic pathways and are developing cell-culture methods that model human metabolic systems. One particularly important issue is how metabolites (the intermediate products formed by enzymes) interact with the genetic material. We need to know exactly what takes place at the molecular level, including covalent binding with and structural changes to specific components of DNA. This work taps the skills and facilities in several related research programs, including the Human Genome and DNA repair projects. (See the April/May 1992 and April 1993 issues of *Energy and Technology Review* for more background on these two programs.)

In assessing the effects of low-level exposure to food mutagens, we make use of Laboratory expertise in accelerator mass spectrometry (AMS). Yet another part of the story is the differences among humans in susceptibility to cancer, which has become our newest effort. In essence, our success in recent years is derived not so much from simply applying standard analytical methods by themselves as from combining biological analysis with state-of-the-art analytical tools available at LLNL to study all aspects of the health risks, ranging from dietary exposure to effects in model systems and humans.

Food Mutagens: The Challenge of Identification

IN the foods that make up the Western diet, the most common mutagens belong to a class collectively called the amino-imidazoazaarenes (AIAs). Not all the known food mutagens are AIAs, but the commonly found ones are. As shown in Figure 2, AIA compounds have one or two aromatic ring structures fused to the imidazole ring. They also have an amino group (NH_2) on the number-2 position of the imidazole ring and can have methyl groups (CH_3) of varying number and location.

Of the list of toxic substances known to be produced during cooking, the most important may well be the AIAs. Also referred to as heterocyclic amines, these compounds are potent mutagens produced at normal cooking temperatures in beef, chicken, pork, and fish when fried, broiled, or grilled over an open flame. The pan residues that remain after frying also have high mutagenic activity, indicating that meat gravies can be a source of exposure. Our research suggests that smoke from cooking muscle meats is mutagenic as well, but any such air exposure is likely to be far less than that from eating the cooked food. Other foods, such as cheese, tofu, and meats derived from organs other than animal muscle, have very low or undetectable levels of AIA mutagens after they are cooked.

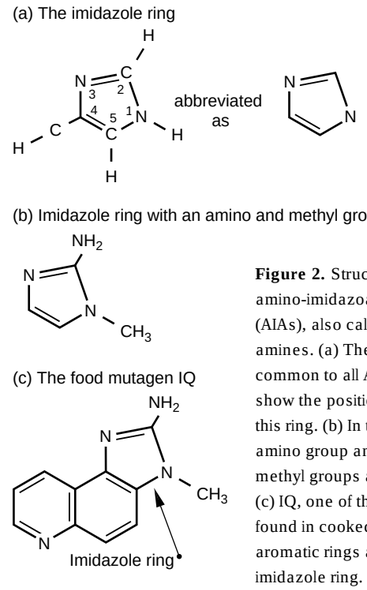


Figure 2. Structure of the amino-imidazoazaarenes (AIAs), also called heterocyclic amines. (a) The imidazole ring is common to all AIAs. Numbers show the position of atoms on this ring. (b) In the AIAs, an amino group and one or more methyl groups are attached. (c) IQ, one of the potent AIAs found in cooked meats, has two aromatic rings attached to the imidazole ring. The mutagenic activity of the different heterocyclic amines varies by several orders of magnitude and can be increased when one or more additional methyl groups are present.

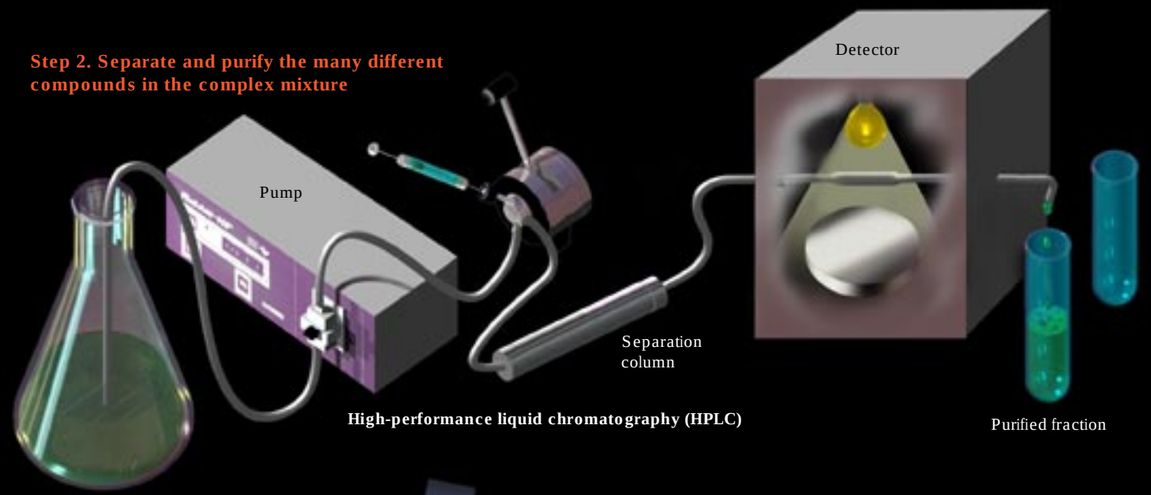
Extraction

Analyzing cooked foods for mutagens requires many different methods (Figure 3). The toxic compounds in food must first be

Step 1. Extract mutagens from cooked food

Solid-phase extraction

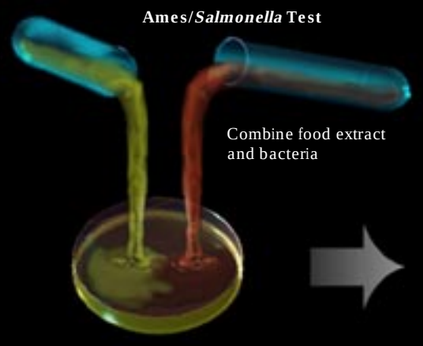
Step 2. Separate and purify the many different compounds in the complex mixture



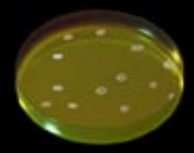
High-performance liquid chromatography (HPLC)

Step 3. Detect Mutagenic Activity

Ames/*Salmonella* Test

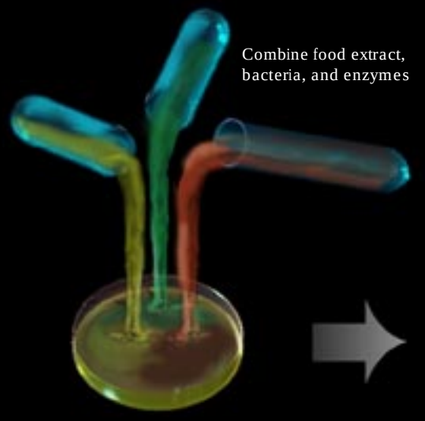


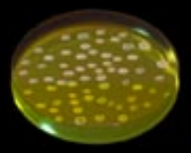
Combine food extract and bacteria



Count revertant colonies (baseline measurement)

Combine food extract, bacteria, and enzymes



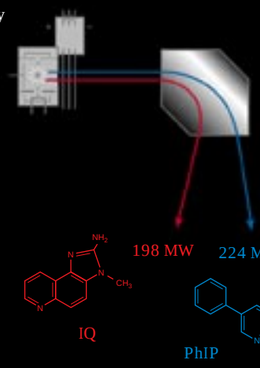


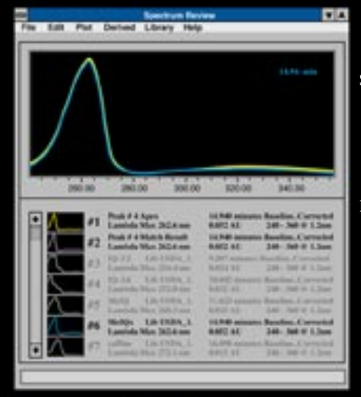
Count revertant colonies

Step 4. Subsequent Characterization

Mass spectrometry

Determine molecular weight (MW) and chemical composition





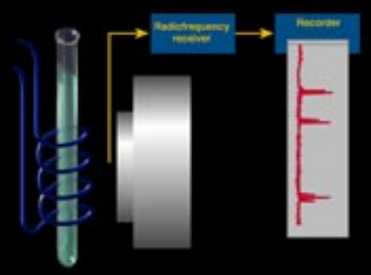
Spectrum review

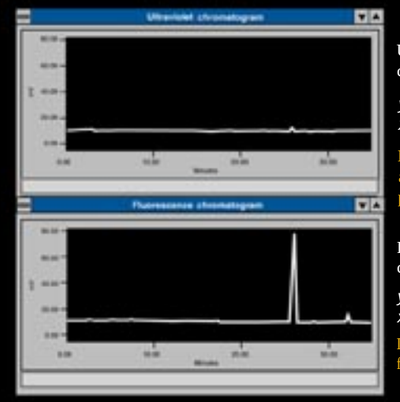
Matching spectra

Confirm mutagen identity

Nuclear magnetic resonance (NMR) spectrometry

Determine definitive structure





Ultraviolet chromatogram

y axis = Absorbance
x axis = Retention time

Detect peak absorbance at a particular retention time

Fluorescence chromatogram

y axis = Fluorescence
x axis = Retention time

Increase sensitivity for fluorescence only

Figure 3. Some of the steps required to extract, separate, purify, and confirm the potency and chemical structure of mutagens in cooked food. These steps show a typical sequence of events during research on a given mutagen. However, the sequence shown here can vary depending on whether our objective is to study a known mutagen or to assess the properties of a new candidate. Each of the steps is described in more detail in the text.

Figure 4. Researcher Cyndy Salmon uses solid-phase extraction to extract a sample by passing it through a series of small cylinders containing small amounts of organic particles.

chemically extracted before purification. Over the years, we and other researchers have dramatically improved on the original extraction techniques that required various acids or mixed organic solvents in multistep schemes.

We now use solid-phase extraction, which is based on a method first described by G. A. Gross in 1990.[4] After homogenizing cooked food in a blender to obtain a uniform sample, we can extract a sample quickly and efficiently by passing it through a series of small tapered tubes containing chemically activated particles (see step 1 in Figure 3 and Figure 4). The small amounts of organic solvents that are needed during this solid-phase extraction generate a minimum of hazardous waste.

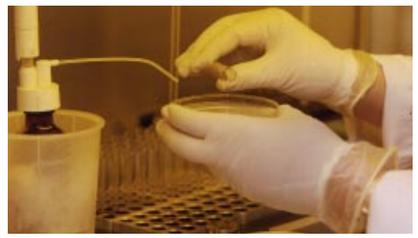
Separation and Purification

We use high-performance liquid chromatography (HPLC) for final separation and purification of the extracted compounds in a food sample (see step 2 in Figure 3). Liquid chromatography is a standard technique in chemistry labs. In HPLC, a liquid mixture is pumped under high pressure through a long, narrow tube filled with fine silica particles. This material differentially retards the passage of different molecular components so that each one exits after a characteristic delay or retention time. Our recent solid-phase extraction method together with HPLC allows excellent quantification from small samples (about a tenth of a hamburger patty, or one bite) and a 1- to 2-day turnaround time for results.

For unknown mutagens, a separation is carried out in several stages. We obtain about 100 fractions at the final stage, where a "fraction" is one portion of the sample material that is captured in a separate vial after exiting the HPLC detector. One fraction at the final stage of separation contains as little as a billionth of the starting material. However, because the extracts from meat and other food products cooked at elevated temperatures are tremendously potent, only a very small sample is needed for the next step—testing for mutagenic potency.

(a)

(b)



(c)

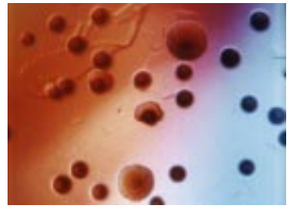


Figure 5. Julie Avila, one of the researchers in the LLNL food mutagen research group, tests mutagens in cooked beef using the Ames/*Salmonella* test. (a) The food sample is added to a mixture containing bacteria, nutrients, and enzymes needed for metabolism, and then (b) poured onto a petri plate. (c) Close up of growing bacterial colonies (called revertants) after 48 hours. Counting the colonies gives us a number that represents the sample's mutagenic activity.

Detection of Mutagenicity

The most widely used detection method for mutagenic potency is the Ames/*Salmonella* mutation test,[1] which is described in more detail in the box on p. 16. This test for mutagenic activity is exquisitely sensitive and relatively inexpensive. It is also convenient because each analysis requires only 48 hours, and many samples can be analyzed in parallel (Figure 5).

The essential point to remember is that the Ames test (step 3 in Figure 3) gives us a number by which we can express the mutagenic *activity* of a given compound or food extract. This number by itself for a single mutagen would have little meaning. However, we now have numbers for most of the known mutagens in cooked foods and for over a hundred additional mutagens from other sources, so we can compare the mutagenic activity of many different structural types. When the Ames test is used during initial screening for new mutagens and carcinogens, it serves as a guide to the chemical purification of biologically active molecules. It can also be used to test and compare the potency of newly synthesized chemicals.

Characterization

Once a mutagen has been detected, we can characterize it further through a variety of analytical methods (step 4 in Figure 3). The type and sequence of tests depend on our objective for a given mutagen (Figure 6). For example, we can routinely determine the molecular weight through mass spectrometry and study the detailed chemical composition (the number of hydrogen, carbon, and nitrogen atoms) by high-resolution mass spectrometry (HRMS). In mass spectrometry, complex compounds are broken up into ionized fragments, which are accelerated through a magnetic field until they strike a detector. Because the path of an ionized fragment through the field is determined by its inertia, we can determine the mass of the various ions by their spatial distribution on the detector. Ultraviolet absorbance spectrometry and fluorescence spectrometry are other identification methods that are often combined with chromatography.

Substantially more effort is required if we want to identify a mutagen for the first time. For an unknown compound, we first need information on the atomic composition and the position of atoms in the molecule. This work requires HRMS and nuclear magnetic resonance (NMR) spectra (step 4 in Figure 3)

Figure 6. Kathleen Dewhirst combines methods, such as gas chromatography and mass spectrometry or liquid chromatography and mass spectrometry, to characterize the food mutagens in cooked meat. Mass spectrometry allows us to determine the molecular weight of a mutagen.

The Ames/*Salmonella* Test: A Key to Our Research

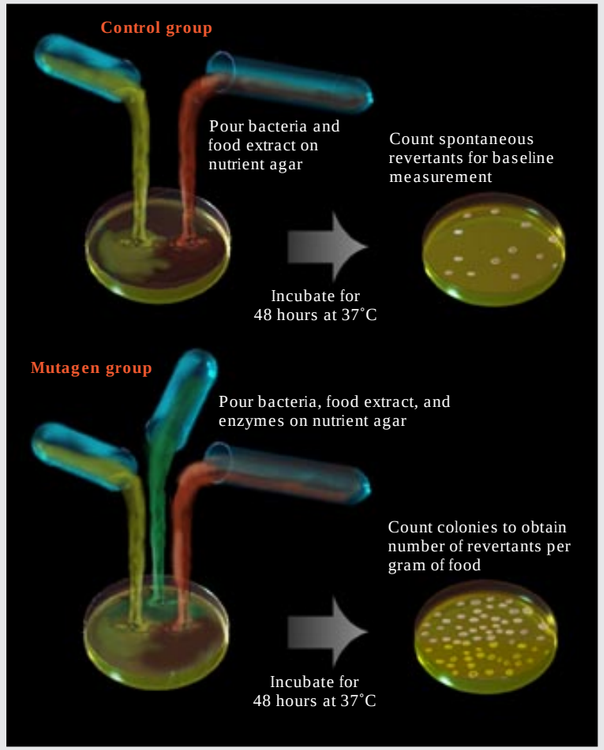
Our success in detecting and identifying mutagens in cooked foods is made possible by the interplay of many different types of chemical analyses, including chromatography and mass spectrometry (Figure 3), and biological methods. The Ames test is an exquisitely sensitive biological method for measuring the mutagenic potency of chemical substances. The Ames test by itself does not demonstrate cancer risk; however, mutagenic potency in this test does correlate with the carcinogenic potency for many chemicals in rodents.

The test was developed in 1975 by Bruce Ames and his colleagues at The University of California at Berkeley. The Ames method is based on inducing growth in genetically altered strains of the bacterium *Salmonella typhimurium*. To grow, the special strains need the amino acid histidine. However, when the chemical agent (mutagen) that is being studied is given to bacteria, some of the altered *Salmonella* undergo mutations. Following a particular type of mutation, the bacteria can grow like the original "wild" (unaltered) strains without histidine. Because the mutant bacteria revert to their original character with regard to the nutrient histidine, they are called "revertants."

The Ames test yields a number—specifically, the number of growing bacterial colonies—which is a measure of the mutagenic activity (potency) of a treatment chemical. This value is often expressed as the number of revertants per microgram of a pure chemical (mutagen) or per gram of food containing that mutagen. Some pure mutagens result in hundreds of revertants per microgram, but many of the substances we have tested from cooked meat produce hundreds of thousands of revertants per microgram. For example, in one strain of bacterium, the PhIP mutagen results in about 2000 revertants per microgram, whereas another cooked food mutagen, IQ, results in 200,000. The illustration at the right shows how a food extract is tested for its mutagenic activity.

In brief, a test begins by placing about 100 million *Salmonella* bacteria in a petri dish containing a nutrient agar lacking histidine. A few bacteria will spontaneously revert in the absence of mutagens. Counting these revertant colonies gives us a baseline against which to check the validity of our complex laboratory procedures. In a separate but essentially identical histidine-deficient petri dish, another batch of *Salmonella* bacteria are given a mutagen plus mammalian enzymes required for metabolism. (Adding such enzymes gives us a more realistic measure of the mutagenicity of a substance for mammals. The enzymes are typically supplied from liver cell extracts of rats given substances to increase levels of metabolizing enzymes.) Revertant bacteria grow into visible colonies. We simply count the colonies (equal to the number of revertants) after a standard time (48 hours) under standard growing conditions (37°C).

Different strains of altered *Salmonella* bacteria are available for the Ames test. The strains vary in sensitivity to specific mutagens. We used two strains, known as TA98 or TA100, for most of our recent work on fried beef and cooked grains. These strains were generously supplied by Bruce Ames.



together with synthesis of all possible isomers. Isomers are two molecules with the same number of atoms and molecular weight but different structures. NMR spectrometry requires the highest quantity and sample purity of all the analytical methods, but it gives us the most definitive information on chemical structure. The exact chemical structure of a given mutagen can be proven by comparing it with a known standard that is synthesized in the laboratory.

After the physical and chemical properties of a mutagen are known, we can use the information to determine whether that mutagen is present in other types of food. This approach gives us a way to determine the dose of a given compound in our diet and to assess the human risk associated with ingesting that compound.

The Major Food Mutagens

Table 2 is a summary of the 14 major mutagens that have been identified in at least one type of heated food to date.[5] Notice that some of the compounds have the same molecular weights. For example, 4-MeIQx and 8-MeIQx are isomer pairs and so are Trp-P-2 and Me-AαC. The ultraviolet absorbance spectra of two different compounds may be identical when they are isomer pairs and differ only, for example, in the position of a methyl group on one of the rings. The similar properties of isomers make them difficult to separate using chromatography. Likewise, other analytic tools do not always differentiate between isomers. Additional mutagenic isomers have been synthesized for most of the food mutagens in Table 2. The presence of isomers means that we need to apply several different criteria for identification purposes because no single property, such as an absorbance spectrum, can uniquely identify all of the mutagens.

The compounds listed in Table 2 are not the only mutagens or carcinogens in food. Researchers at LLNL and elsewhere have identified other biologically active compounds, including additional aromatic amines, nitrosamines, and hydrazines. However, the heterocyclic amines we have been investigating are among the most abundant and potent substances detected to date. Because of their presence in cooked meats that are common in Western diets and their association with certain types of cancer in laboratory animals, they warrant detailed investigation.

Table 2. Major mutagens that have been identified in at least one type of heated food, such as fried beef or fish. The names of mutagens first identified at LLNL are in color.

Short name	Chemical name	Molecular weight
Phe-P-1	2-amino-5-phenylpyridine	170
TMIP	2-amino-n,n,n-trimethyl-imidazo[4,5-f]-pyridine	176
AåC	2-amino-9H-pyrido-[2,3-b]-indole	183
Glu-P-2	2-aminodipyrido-[1,2-a:3′,2′-d]-imidazole	184
Trp-P-2	3-amino-1-methyl-5H-pyrido[4,3-b]-indole	197
Me-AåC	2-amino-3-methyl-9H-pyrido[2,3-b]-indole	197
IQ	2-amino-3-methyl-imidazo[4,5-f]-quinoline	198
IQx	2-amino-3-methyl-imidazo[4,5-f]-quinoxaline	199
Trp-P-1	3-amino-1,4-dimethyl-5H-pyrido[4,3-b]-indole	211
4-MeIQ	2-amino-3,4-dimethyl-imidazo[4,5-f]-quinoline	212
8-MeIQx	2-amino-3,8-dimethyl-imidazo[4,5-f]-quinoxaline	213
4-MeIQx	2-amino-3,4-dimethyl-imidazo[4,5-f]-quinoxaline	213
PhIP	2-amino-1-methyl-6-phenyl-imidazo[4,5-b]-pyridine	224
4,8-DiMeIQx	2-amino-3,4,8-trimethyl-imidazo[4,5-f]-quinoxaline	227

Food Mutagens: The Cooking Makes a Difference

COOKING practices can cause large variations in the total mutagenic activity and in the amount of specific mutagens present in muscle-containing foods. For example, the amount of mutagens in a cooked hamburger from a restaurant varies considerably from one vendor to another and is often several-fold *lower* than that in a hamburger prepared in our laboratory (and presumably at home). The variation has much to do with the details of food preparation, such as cooking temperature and cooking time. It is becoming increasingly clear that there can be many different routes and rates of formation for the different mutagens we are investigating. Thus, a major concern is to identify the precursors and specific reaction conditions that lead to the formation of mutagens during cooking. With this information, it may be possible to devise strategies to reduce or prevent the formation of mutagens.

Precursors

The reactions that produce mutagens in cooked food are not merely the random coalescence of small fragments. We now know that the heterocyclic amines can be formed from single amino acids (the building blocks of proteins) or proteins when these precursors are heated alone. However, the temperatures required to produce mutagens from amino acids or proteins by themselves are higher than those normally used in cooking.

Muscle meats contain creatine and creatinine. At more typical cooking temperatures (greater than 150°C), one or both of these two precursors react with the free amino acids and, in some cases, sugars to form a series of heterocyclic amines more easily.

Modeling the Formation

We have modeled the formation of the important mutagen, PhIP (pronounced "fip"), starting with the amino acid phenylalanine mixed with either creatine or creatinine, both of which are found naturally in animal muscle. When phenylalanine and creatine are mixed in the proportion normally found in raw beef and dry heated at 200°C, PhIP is produced in amounts comparable to those found after cooking beef. Figure 7 shows the structures of phenylalanine and creatine and of the PhIP molecule that is produced.

We have modeled the formation of several other food mutagens in additional laboratory experiments. For example, the mutagen IQ can be formed with creatine, creatinine, and any of four different amino acids, again suggesting many different possible routes of formation.

Model reactions can help us identify new mutagens as well. In one case, dry heating three precursors known to be present in meat led us to identify a mutagen with two amino and two methyl groups and a molecular weight of 244. However, the presence of this new mutagen in food has not been verified.

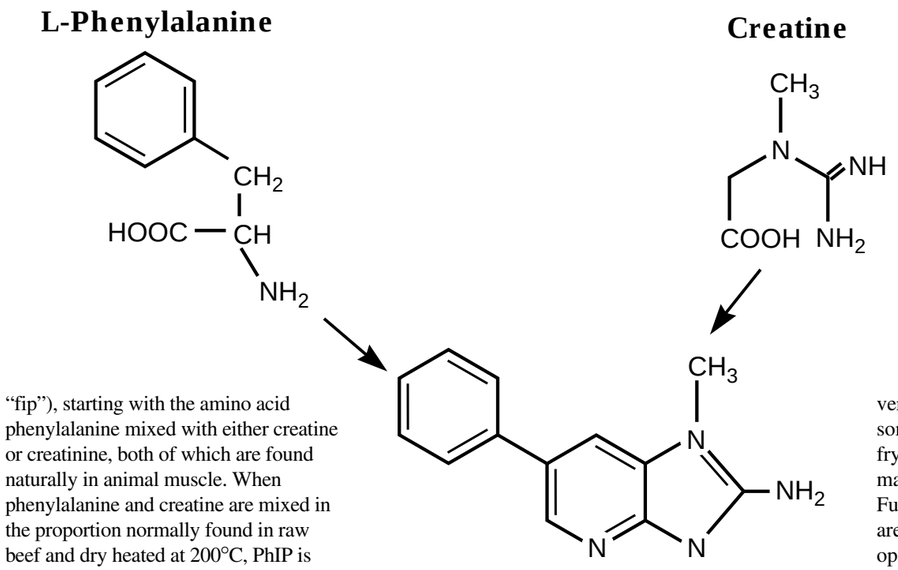


Figure 7. Modeling the formation of the potent mutagen PhIP. We combined two precursors, phenylalanine and creatine, in amounts naturally found in raw beef. After simple dry heating, PhIP was produced in yields comparable to those we obtain in beef after the cooking process. We have also labeled the two precursors with heavy isotopes to track the incorporation of specific atoms within each precursor molecule into the PhIP molecule. Such work shows unequivocally the source of atoms that make up the mutagenic product.

Variations in Cooking

During the actual cooking of meat patties, water and precursors move to the hot, drying contact surfaces of the meat where reactions occur. Such migration, with water serving as the transport vehicle, may account for the concentration of precursors near the meat surface, which we have observed in several investigations. However, different cooking practices can lead to very different results. For example, some mutagens are produced at all frying temperatures, whereas others may require higher temperatures. Furthermore, when hamburger patties are grilled at high temperature over an open flame, we can account for less than 30% of the mutagens in the meat. When cooking over an open flame, polycyclic aromatic hydrocarbons (different from AIA food mutagens) arise from fat that drips from the meat—this is an entirely different mechanism than those that produce heterocyclic amines from heated muscle tissue itself. Thus, the formation of mutagens is complex and highly dependent on the details of cooking.

Preparation Principles

Given this complicated picture, what statements about food preparation can we make with any certainty? Here is a summary of some of the important things we have learned about the cooking process:

- *Food mutagens can be produced both with and without water present.* Early reports suggested that water is essential to produce food mutagens. In later studies, dry heating actually gives a greater percentage of certain types of mutagens compared with aqueous heating. We know, for example, that the mutagen PhIP is formed relatively efficiently in dry heating reactions. We have also found that water tends to inhibit the formation of IQ-type mutagens.
- *Microwave pretreatment of meat can reduce the formation of heterocyclic amine mutagens.* When meat is microwaved for a few minutes, a clear liquid is released, which contains many of the precursors of mutagens. When the resulting liquid is drained off before frying, our studies show that mutagenic activity, as measured by the Ames test, and the amount of

heterocyclic amine are 90 to 95% lower than they are in meat samples that are not pretreated by microwave cooking.[6] The box below discusses this and other methods that have been tested to reduce the formation of mutagens.

• *Different cooking methods produce quite different results.* In general, frying, broiling, and flame grilling muscle meats produce more heterocyclic amines and mutagenic activity than other methods. Stewing, steaming, and poaching produce little or no mutagenic activity. Roasting and baking have variable responses.

• *Heating temperature is extremely important as is the time of cooking at a given temperature.* Our extensive findings on this important topic are best discussed according to the type of food product.

Cooked Muscle Meats

Fried beef patties appear to be the most commonly eaten cooked meat with the highest mutagenic activity. Because of the high intake of fried beef (based on surveys from the U.S. Department of Agriculture and the Department of Health, Education, and Welfare), this food may be the most important single source of heterocyclic amines in the typical American diet. However, several other popular cooked meats, including fish, chicken, and pork, have been shown to produce a potent response in the Ames test.

Of the several different heterocyclic amine mutagens now identified, we wanted to know which ones are most important (that is, most abundant by mass) in cooked muscle meats. To help answer this question, we compared the results of many studies from LLNL and elsewhere. Specifically, we compared the mass percentages of different mutagens in cooked muscle meats, including fried beef, broiled fish, and commercially prepared beef extract. We found that the results were generally consistent among different laboratories even when different analytical methods were used.

First, we did not detect significant levels of three mutagens, Trp-P-1, Trp-P-2, and Glu-P-1, in any of our meat samples. Second, we found that four compounds, IQ, 8-MeIQx, 4,8-DiMeIQx, and PhIP, contribute about 80% of the mutagenic activity in the cooked muscle foods that were studied. Third, we found that PhIP alone can account for a startling 83 to 93% of the mass of these four mutagenic compounds. Clearly, the analysis of PhIP is important because it appears to be, by far, the most abundant heterocyclic amine by mass in commonly eaten cooked meats. Because PhIP is as carcinogenic as the other mutagens, its analysis becomes even more essential.

We examined the production of PhIP and other mutagens in beef at different cooking temperatures and times. The box at the right gives the details on how we prepare our fried beef. Figure 8 shows the mutagenic activity, as measured by the Ames test, of all the mutagens combined in a gram of beef

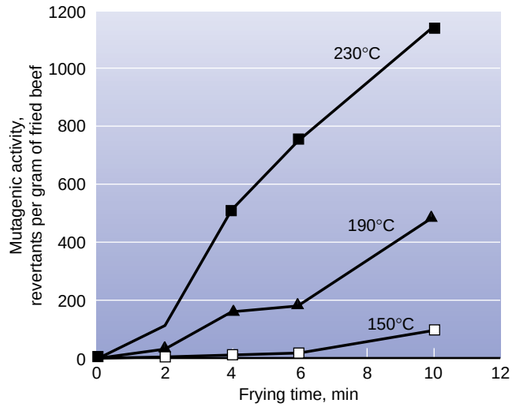


Figure 8. A graph of the mutagenic activity in beef patties fried at three different temperatures. The essential point is that mutagenic activity increases with both frying temperature and time.

How We Fried the Burgers We Studied

One major difficulty in our dose- and exposure-assessment work is that the content of mutagens can vary widely even in the same kind of food product when it is obtained from different suppliers or prepared by different restaurants. Although the relative amounts of the heterocyclic amines are generally consistent among different studies and laboratories, the precise amount of mutagen per gram in a given cooked food can span a tenfold range.

Hamburgers from fast-food restaurants generally have considerably lower levels of mutagens than those prepared at home. This result is probably due to the fact that many fast-food restaurants cook their meat at moderate temperatures on a grill or over open flames for a short time. Because the meat patties are thin, the products are not generally overcooked.

Because food-preparation practices vary, over the years we have attempted to approximate a range of cooking practices that are common in American households. In various experiments, foods were pan fried, oven broiled, baked, boiled, stewed, grilled over coals, or left raw. However, for the studies on red meat reported in this article (see Table 3), we purchased ground beef, sold as containing 15% fat, from a local market. We formed the meat into patties weighing 100 grams (a little less than a quarter of a pound) and fried them on a commercial, electric, stainless-steel griddle for 2 to 10 minutes per side and at surface temperatures of 150, 190, or 230°C. We monitored the griddle surface with a digital probe thermometer. After the meat was cooked, it was homogenized in a blender to produce a uniform sample. Samples were frozen at –4°C until extraction for subsequent testing and analysis.

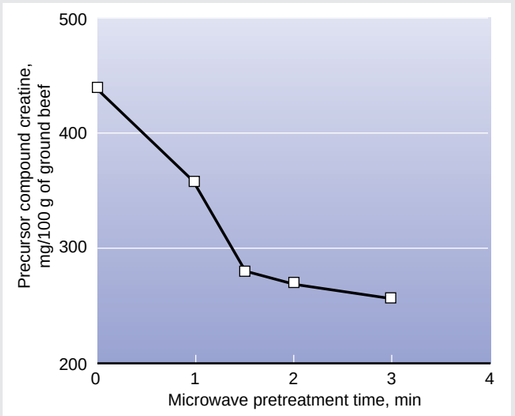
Can the Mutagens in Cooked Beef Be Reduced?

Since mutagens were first observed in cooked meats, researchers in several different laboratories have explored various ways to reduce the amounts produced during food preparation. They have found that mutagenic activity can be lowered by adding antioxidants, soy or cottonseed flour, tryptophan, and various other food additives or sugars either alone or with starch. However, none of these additives is widely used commercially or at home. Consumer acceptance and possible changes in the taste, texture, and nutritional content of the cooked food need to be explored further.

Surveys have shown that more than 90% of American homes have a microwave oven. As a practical way to reduce the mutagen and fat content of beef, we studied microwave pretreatment of hamburger for various times before conventional frying either at 200 or 250°C for 6 minutes per side. Our tests used a standard commercial microwave oven set at 80% power for 0 to 3 minutes. The results were dramatic.

We found that the mutagen precursors in hamburger (creatine, creatinine, amino acids, and glucose), water, and fat were reduced up to 30% in the microwaved patties. The graph shows the amount of creatine remaining in the meat as a function of microwave pretreatment times. The fairly rapid loss of water-soluble mutagen precursors and fat takes place in the clear liquid that is released after microwaving. When this liquid is discarded before frying, mutagens in the cooked meat are reduced up to approximately 90% following frying, as shown in the table.

How is it possible that 90% of the mutagens disappear when the precursors are reduced by only 30%? The difference can be explained by second-order reaction kinetics. For example, if two reactants are needed, and each is reduced by 30%, then the product would be reduced by about 50%. If three reactants are required and all are reduced by 30%, the product would be reduced by 70 to 80%. It is also possible that some threshold level of precursor is necessary to produce a mutagenic response or that some inhibitor is formed after microwave pretreatment. As with other techniques to reduce mutagen content, the palatability of food may ultimately govern consumer acceptance of microwave pretreatment.



Measured mutagenic activity, from the Ames test, in beef patties pretreated in a microwave oven and then fried for 6 minutes at 200 or 250°C.

Microwave time, min	Mutagenic activity from the Ames test, revertants per gram	
	200°C	250°C
0	450	1400
1.0	220	369
1.5	130	216
2.0	47	67
3.0	16	41

patty fried at 150°, 190°, and 230°C. We found no detectable heterocyclic amines after frying at 150°C for 2 to 4 minutes. In general, increasing either the temperature or time of cooking (specifically, frying on a solid metal restaurant-type grill) causes a dramatic increase in both the mutagenic activity and the total amount of mutagens

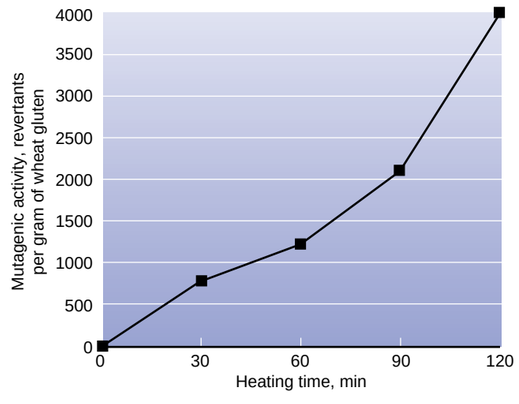


Figure 9. The mutagenic activity of wheat gluten increases dramatically when heated at 210°C for up to 2 hours. This potent response tells us that one or more highly mutagenic chemicals, still unidentified, are formed with continued cooking at high temperature.

Table 3. Content of four different mutagens in fried beef patties (expressed as nanograms of mutagen per gram of beef) cooked at different times and temperatures.

Mutagen	Cooking time per side, min	Cooking temperature of grill, °C 150	190	230
IQ	2	none	0.1	none
	4	none	0.1	0.15
	6	0.1	0.45	0.6
	10	0.1	0.85	0.7
8-MeIQx	2	none	0.1	0.7
	4	none	0.25	0.4
	6	0.2	1.3	5.6
	10	0.6	1.3	7.3
4,8-DiMeIQx	2	none	none	1.6
	4	none	0.1	0.15
	6	0.2	0.55	1.2
	10	0.4	1.1	1.0
PhIP	2	none	none	1.3
	4	none	0.15	1.3
	6	0.25	1.9	7.8
	10	1.8	9.8	32.0

produced, especially PhIP and 8-MeIQx. For the most part, as shown in Table 3, the amount of individual mutagens in fried beef increases proportionately with the cooking temperature. A clear exception to this trend is the compound PhIP, which is produced at much greater concentrations at higher temperatures relative to the other mutagens we have studied. When the cooking temperature and time are increased, the PhIP content of fried beef patties increases nearly exponentially.

Mutagens from Grain?

We also recently used the Ames test to assess the mutagenic activity in many heated foods derived from grain products. Our studies include cooked breads (white, pumpernickel, crescent rolls, and pizza crust), breadsticks, heated flour from many different grain sources, breakfast cereals, graham crackers, and meat-substitute patties after frying. These foods were either tested as purchased without additional cooking (for example, graham crackers and a grain beverage powder) or were cooked according to package instructions. In some studies, we deliberately overcooked the grain products for twice the cooking time at the specified temperature setting to see if the mutagen content would increase with continued cooking, as it does in muscle meats.

Our studies generally demonstrate increased mutagenic activity in grain foods with cooking time, but the exact composition of the food is important. For example, when wheat gluten (the protein in wheat seed) is heated alone at 210°C in a beaker, it shows a potent, time-dependent mutagenic response (Figure 9). Because breadsticks are high in wheat gluten, they also show some activity when heated normally and much higher activity when overcooked.

In fact, the mutagenic activity of breadsticks cooked for double the regular heating time is 20% that of a hamburger fried 6 minutes per side at 210°C. In all cases, overcooking grain foods led to much higher mutagenic activity. Cooked garbanzo bean flour and the grain beverage powder, which we tested as purchased, had relatively high mutagenic activity. Cooked rice and rye flour (containing no gluten), on the other hand, showed no detectable activity, and rice cereal showed very little. Fried tofu (soy bean curd) was not mutagenic, and the measured level of activity in meat-substitute patties (which are made from vegetable proteins) after frying was about 10% or less than that of a beef patty cooked under the same conditions.

Table 4 summarizes the mutagenic activity, as measured by the Ames/*Salmonella* test, for a variety of cooked-grain food products. The results are expressed as mutagenic *activity* from the Ames test, so they cannot be directly compared with those in Table 3. (Recall that the numbers in Table 3 represent a different measure, namely the content by weight of individual mutagens expressed as nanograms of mutagen per gram of beef.) Because we do not yet know the identity of the mutagens present in cooked grain products, we cannot provide their content by weight. However, to allow for some comparison between cooked grains and meat, we have included the values of mutagenic activity for hamburger cooked for three different times at the end of Table 4.

Overall, the level of mutagenic activity measured in heated nonmeat foods is lower than that in cooked meats. It is important to recognize that the cooked grains we studied lack the creatine and creatinine levels that explain the formation of mutagens in muscle meats during cooking. We are currently investigating the question of why foods high in gluten are quite mutagenic in the absence of creatine and creatinine. We suspect that the amino acid, arginine, can substitute for the creatine and creatinine precursors found in meat, but it may be a less

Table 4. Mutagenic activity of nonmeat food products (expressed as the number of revertants [mutants] per gram from the Ames/*Salmonella* test using the TA98 strain of bacteria). Results for hamburger are given for comparison.

Food sample	Mutagenic activity, revertants per gram
Flour from plant sources heated to 210°C for 60 minutes	
Chemical-grade gluten	1330
Food-grade wheat gluten	970
Cornmeal	180
Garbanzo flour	1890
Teff flour	420
Rice flour	none detected
Rye flour	none detected
Wheat flour for bread	320
Cooked food samples tested as purchased or cooked as directed	
White bread	2
Pumpernickel	6
Breadsticks	6
Crescent rolls	1
Pizza crust	3
Graham crackers	4
Grain beverage	320
Food samples cooked double the time directed	
White bread	5
Pumpernickel	28
Breadsticks	40
Crescent rolls	4
Pizza crust	8
Toasted breakfast cereals tested as purchased	
Rice-based	2.2
Corn-based	4.4
Wheat-based (various samples)	0 to 8.8
Commercial meat substitutes fried at 210°C for 6 minutes per side	
Gluten-based patties (various samples)	6 to 9.4
Tofu	none detected
Falafel	2.3
Tempeh burger	23
Tofu burger	non detected
Soy-based patties	6.6
Gluten, wheat, teff-based patties (230°C)	30
Hamburger fried at 210°C for 6 minutes per side	220
Hamburger fried at 230°C for 6 minutes per side	800
Hamburger fried at 250°C for 6 minutes per side	1400

efficient mutagen precursor in cooked grain products.

Before we can evaluate the risk associated with cooked grains, we need to determine the mass of mutagens in each food and to identify the specific mutagenic compounds that are present. Except for very low levels of PhIP in wheat gluten (accounting for only 4% of its mutagenic activity), our analysis did not reveal any of the other mutagens found in cooked meat or listed in Table 2. Because the mutagens in cooked grain appear to be as potent as the heterocyclic amines—and such potency is unusual, we suspect that the mutagenic compounds may be new heterocyclic amines similar to those we have found in cooked meats. However, more work needs to be done before we understand the entire picture.

What About Fumes?

Some studies have suggested the possibility of an increased risk of respiratory tract cancer among cooks and bakers. When foods rich in protein are heated, the fumes that are generated sometimes contain many different known carcinogens, including polycyclic aromatic hydrocarbons and heterocyclic amines. Working with colleagues at the University of California at Davis, we recently studied the mutagenicity of fumes generated when beef is fried at high temperatures.

We collected smoke from cooking by using a special sampling system consisting of a condenser, Teflon filters, and absorbent tubes containing polyurethane foam and a resin. We found that the main volatile compounds generated during frying were alcohols, alkanes, aldehydes, ketones, phenols, and acids. Their presence—we measured 34 different components—may account for much of the toxicity of fume samples in bacterial tests. Two mutagens, PhIP and AαC, were the most abundant of the heterocyclic amines we measured in smoke, with AαC accounting for 57% of the total weight of mutagens in the recovered samples. However, even though AαC seems to be the most volatile of our quantified heterocyclic amines in smoke, its actual contribution to the mutagenicity of fumes is negligible because its mutagenic potency is lower than that of some other heterocyclic amines in smoke. We also detected significant levels of MeIQx and DiMeIQx.

In a modified Ames test, one that is much more sensitive than our standard assay and uses two different strains of bacteria, the fried meat extracts had 30,700 revertants per gram (see box, p. 16 for a definition of "revertants"), whereas the fumes produced by frying had 10,400 revertants per gram of fried meat. Thus, the fumes generated during the cooking of meat represent about one-third of the mutagenic activity measured in the fried meat itself. It is important to recognize that the amount of mutagens inhaled is very low compared to consuming solid, cooked meat. Nevertheless, the presence of toxic compounds in meat fumes, even at relatively low levels, could pose some risk to food preparers who are exposed to them for long periods over many years.

Cook to Manage Mutagens

Our research on food mutagens is not specifically designed to generate practical advice for diet- and health-conscious individuals. Many questions remain unanswered in this highly complex field of investigation. Although food mutagens are extremely potent, our preliminary estimates of risk are not alarming primarily because of their low concentrations in food. Nevertheless, the amount of mutagens ingested can be reduced by choice of diet and by modifying cooking practices.

Cooking Tips Summary

- Fried beef has very high mutagenic activity. Its popularity suggests that this food may be the most important source of heterocyclic amines in the typical Western diet.
- Most, but not all, of the mutagenic activity in fried beef can be accounted for by known heterocyclic amines. The single mutagen PhIP accounts for most of the combined mass of mutagens in fried beef cooked well-done.
- The fumes generated during the cooking of beef have about one-third the mutagenic activity measured in the fried meat itself. Occupational exposure over long periods could pose some risk, but probably much less than that from consuming the meat.
- The fat content and thickness of meat have little effect on mutagen content, whereas the method and extent of cooking have major effects. Frying, broiling, and barbecuing muscle meats produce more heterocyclic amines and mutagenic activity, whereas stewing, steaming, and poaching produce little or no mutagenic activity. Roasting and baking show variable responses.
- Both cooking temperature and time can be manipulated to minimize the formation of mutagens. Increasing the frying temperature of ground beef from 200 to 250°C increases mutagenic activity about six- to sevenfold. Reducing cooking temperature and time can significantly lower the amounts of mutagens generated and subsequently consumed in the diet.
- Microwave pretreatment of meat, followed by pouring off the clear liquid before further cooking, can substantially reduce the formation of heterocyclic amine mutagens, even if the meat is cooked well-done.
- Most nonmeat foods, including cooked grain products, contain lower levels of mutagens than cooked meats.

At least in rodents, we know that food mutagens trigger cancer in several different target tissues, such as the liver, colon, breast, and pancreas. In a follow-up installment in *Science and Technology Review*, we will address the health risks to humans that may arise from exposure to heterocyclic amines. For this intriguing part of the story, we will show how these highly toxic compounds can react with the most critical macromolecule of all, DNA. With a connection established between food mutagens, DNA damage, and the potential for cancer, we will then try to make sense of what all the numbers on mutagenic activity and mutagen content in food mean for the average person.

Key Words: Ames/*Salmonella* assay; amino-imidazoazaarenes (AIAs); carcinogen; DNA adducts; heterocyclic amines; high-performance liquid chromatography (HPLC); mutagens—airborne, in cooked foods, in fried beef; mutagenicity; 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP); 2-amino-3-methyl-imidazo[4,5-f]quinoline (IQ); 2-amino-3,8-dimethyl-imidazo [4,5-f]quinoxaline (MeIQx).

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In more than 147 professional publications, James Felton has explored the role of diet in carcinogenesis and mutagenesis. He has been a part of the Laboratory's research on food mutagens since it began 17 years ago and has led it for the past 8 years.